Development of Real-Time PCR Assays for the Quantitative Detection of Herpesviruses

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ABSTRACT

Luciana E. Leopold: Development of Real-Time PCR Assays for the Quantitative Detection of Herpesviruses
(Under the direction of Dirk P. Dittmer, Ph.D.)

Kaposis's sarcoma-associated Herpesvirus (KSHV) and Human Epstein-Barr virus (EBV) are gamma herpesviruses that can cause serious complications in immuno-compromised patients. Quantitative detection is critical not only as a diagnostic tool in patients, but also as a method for basic herpesvirology research. Here the development and validation of four real-time PCR assays to quantify viral load of KSHV and EBV in samples is described. The assays are: individual viral load assays for KSHV and EBV, and high-throughput viral load screens for KSHV and EBV. The assays were developed for validation of animal models, drug response studies, basic virology research and medical diagnostics. The data indicate that these are accurate, consistent, and cost-effective assays for the detection of KSHV and EBV infection in various kinds of samples.

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I would like to thank my mentor Dirk P. Dittmer, Ph.D. and the members of the Dittmer lab for all of their advice, assistance, and kindness.

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LIST OF ABBREVIATIONS

DHHS	United States Department of Health and Human Services
EBV	Epstein-Barr virus
HHV-8	Human herpesvirus 8
IPTG	isopropyl-beta-D-thiogalactopyranoside
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
LANA	Latent nuclear antigen
LCL	Lymphoblastoid cell line
LoD	Limit of detection
MCD	Multicentric Castleman's disease
NTC	Non-template control
OHARA	Oral HIV/AIDS Research Alliance
ORF	Open-reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEL	Primary effusion lymphoma
QPCR	Quantitative Real-time PCR
RPMI	Roswell Park Memorial Institute medium
SAP	Shrimp alkaline phosphatase
X-Gal	5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside

I. INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8 (HHV-8) and Epstein-Barr virus (EBV) or human herpesvirus 4 (HHV-4) are both members of the viral subfamily of gamma herpesviruses. KSHV is an ongogenic herpesvirus known to cause Kaposi's sarcoma (KS) (2,6),and is associated with multicentric Castleman's disease (MCD) (14) and primary effusion lymphoma (PEL) (5). Like KSHV, EBV has, aside from causing infectious mononucleosis, been shown to be oncogenic and involved in the development of various malignancies, i.e. African Burkitt's lymphoma, B-cell lymphoma, nasopharyngeal carcinoma, and in some cases of Hodgkin's disease and gastric cancer. (19) Both KSHV and EBV have their most severe impacts on immuno-compromised patients, particularly AIDS patients, as it is believed that the host response is weakened in these patients and reactivation of the virus can occur, leading to lytic replication and malignancy. Early diagnosis of reactivation of these viruses after initial latent infection is key to disease management so that anti-viral therapy can begin as soon as possible. Accurate, quantitative methods are needed to detect the viruses. Aside from the obvious clinical relevance that such quantitative assays would have, they could also broadly apply to basic research into these viruses.

Amplification of viral DNA by polymerase chain reaction (PCR) is an effective method for detecting viral DNA. Qualitative PCR assays have in the past been very useful for screening samples for the presence of viral DNA (11, 27). For applications as those mentioned above – detecting the difference between lack of infection, latent

infection, and reactivated infection – quantitative analysis must be used. Quantitative real-time PCR can be utilized to accurately and specifically measure differences in viral titer. In this study, we show that the technique can be applied not only to different sample types, but shaped into assays with broad applications.

The four assays that have been developed are as follows: an individual sample EBV viral load assay, an individual sample KSHV viral load assay, a high-throughput EBV viral load screen, and a high-throughput KSHV screen.

The individual sample EBV assay was developed to detect EBV from a novel sample – a patient's gargle – for the OHARA project. This kind of sample is easy and painless to extract from a patient, and could be used to quickly diagnose oral EBV infection. A diagnostic tool like this would be very useful in medicine as it is quick, simple, and cost-effective.

The individual sample KSHV viral load assay was developed to evaluate a novel mouse model for KSHV. The assay was used to determine levels of virus in different organs. A model such as this would be very useful in the field of KSHV research, not only for studying the molecular dynamics of KSHV, but also for the testing of cures, vaccines, etc.

The high-throughput EBV viral load screen was developed to screen drug-treated lymphoblastoid cell lines (LCL) for EBV reactivation. In this study, numerous drugs are to be tested in different cell lines under different conditions, and the only way to do this is with a high-throughput screen. Quantification is necessary because the cell/drug/condition combinations with the highest response will be studied in more detail.

The high-throughput KSHV viral load screen was intended for use in studies of KSHV episomal loss. KSHV maintains itself as an episome in the host cell, tethering its own circular DNA to the chromosomes of the host via the latent nuclear antigen (LANA). In culture, 99% of the infected cells maintain the episome latently, while 1% spontaneously loses the episome. It is this 1% population that is of interest in this study. Using quantitative real-time PCR to evaluate viral load of individual samples, we will select those with lower viral load to grow up. This process will continue until a population of cells with the lowest possible viral load is isolated. Once this population has been isolated, a number of studies can be done, i.e. deducing which genes are controlling the spontaneous shedding of the episome, tumorigenesis studies, and merely a statistical analysis of the data collected in this study could shed much light on how KSHV maintains itself in the host. Understanding of how the virus is spontaneously lost or shed will be a key piece of work that will not only further the knowledge base of KSHV, but may provide a pathway for development of a cure.

All of these methods were developed for separate purposes, but provide a coherent package of viral load analysis of the herpesviruses EBV and KSHV for use in future research endeavors. We will validate these assays using the parameters of linearity, precision, dynamic range, sensitivity, and specificity.

II. METHODS

Primers. Primers U6, LANA78, K6, ERV3, Hel, EBNA3c and Alu (Table 1) were designed from gene sequences found in BLAST database (Figure 1) and purchased from MWG Biotech, Inc. (High Point, NC). Initial concentration of each primer is 100pmol/μL in 0.1x TE.

Positive Control. As a positive control, synthetic oligonucleotide targets were designed for U6, LANA78, K6, ERV3, Hel, and EBNA3c. Each oligonucleotide target was designed from the gene sequence in the BLAST database (Figure 1) and purchased from MWG Biotech, Inc. (High Point, NC). Initial concentration of each oligonucleotide target is 100pmol/μL. Each oligonucleotide was diluted from 100 pmol/μL to $1x10^{-5}$ pmol/μL. Using an approximation of Avogadro's number, 6×10^{23} molecules/mol, we then conclude that each diluted oligonucleotide target contains 6×10^{6} molecules/μL. In the individual sample assays for KSHV and EBV, eight five-fold serial dilutions of this oligonucleotide target stock were made in order to construct a standard curve with eight positive control samples. The resulting concentrations and copy numbers are shown in Table 2. For each experiment, the primers used were matched with the oligonucleotide targets used. For each set of primers, an oligonucleotide mix of the previously mentioned concentrations was made up. Because exact copy number can be calculated from QPCR results using these oligonucleotide targets, we use these to construct a standard curve for our assays, and to calculate exact copy number contained in each sample.

QPCR Set-up. For all four assays developed, a 'master mix' of primer, Lightcycler® 480 SYBR Green I Master (Roche), and sterile water. SYBR Green I Master not only contains all of the components necessary for PCR besides primer, but also contains the dye SYBR Green, which will bind to amplified DNA and fluoresce, allowing us quantitatively detect levels of fluorescence, and therefore of levels of target in the sample being analyzed. For all assays where an NTC (non-template control) was used as a negative control, we used sterile H_2O.

Individual sample assay for EBV. The 'master mix' created for this assay utilizes the ERV, EBNA, and Hel primers, each at a concentration of 5pmol/μL. The primer:water:SYBR ratio is 1:49:100, and in this case we use 5μL primer, 245μL water, and 500μL SYBR for each plate run. Reactions are pipetted into 96-well plate (Bio-Rad Hard Shell 96-well microplate). After pipetting, each reaction contains 22μL of 'master mix' and 8μL of sample. Serial dilutions for the standard curve are also run on the plate. Serial dilutions are pipetted in strip tubes (GeneMate 8-strip PCR tube w/o cap, ISC Bioexpress) by the robot before transfer to the 96-well plate.

Individual sample assay for KSHV. Conditions are the same as above, except the primers used are ERV, LANA78, and K6. ERV and LANA78 are at a concentration of 5pmol/μL, while K6 is at a concentration of 10pmol/μL.

High-throughput EBV screen. . The 'master mix' created for this assay utilizes the ERV and EBNA primers, each at a concentration of 5pmol/μL. The primer:water:SYBR ratio is 1:24:50, and in this case we use 20μL primer, 480μL water, and 100μL SYBR for each plate run. Reactions are pipetted into a 384-well plate (Roche). After pipetting, each reaction contains 6μL of 'master mix' and 4μL of sample.

High-throughput KSHV screen. The 'master mix' created for this assay utilizes the U6 and LANA78 primers, U6 at a concentration of 0.5pmol/μL and LANA78 at a concentration of 1pmol/μL. In this case we use 360μL primer, 324μL water, and 756μL SYBR for each plate run. Reactions are pipetted into a 384-well plate (Roche). After pipetting, each reaction contains 6μL of 'master mix' and 4μL of sample.

Robot and QPCR settings. The MWG RoboGo! pipetting robot was used to set up the reactions. Robot parameters for the individual samples assays for EBV and KSHV are shown in Appendix A. Robot parameters for the high-throughput screens for EBV and KSHV are shown in Appendix B. QPCR for the individual sample assays for EBV and KSHV was run in the MJ Research Opticon 2 Continuous Fluorescence Detection System. A standard 45-cycle reaction was run with a 5 minute ramp to 95°C, then 15 seconds at 95°C, then 30 seconds at 62°C. QPCR for the high-throughput screens for EBV and KSHV was run in the Roche Lightcycler® 480 System for 50 cycles (45 of them are read), with a pre-amplification ramp of 5 minutes at 50°C, then 5 minutes at 95°C, and an amplification of 15 seconds at 95°C, then 30 seconds at 62°C, as well as a melting curve.

Target (DNA) isolation. Samples used in each of the four assays varied greatly in nature, so specific DNA isolation protocols were developed for each.

Individual sample assay for EBV. Samples from gargle were used in this assay. Subjects were given 10mL of sterile PBS in a sterile wide-mouth 50mL conical tube. They were asked to gargle for approximately one minute, and then expectorate into the tube. Samples were then combined, and approximately 7mL of the resulting mixture transferred to 12mL conical tubes. Pellets were formed by spinning in a centrifuge at 400

x g for 10 minutes. Most of the supernatant was decanted. The DNA from the resulting pellet was extracted using Promega Wizard® SV Genomic DNA Purification System (Table 4) with the protocol for isolating genomic DNA from cells using a microcentrifuge. This was then transferred into Thermo Fischer Scientific Matrix Storage Tubes (Hudson, NH) for QPCR use. In some cases, samples of gargle from patients were supplemented with known EBV-positive cells from the cell line B95-8. These were simply added to the 12mL conical before pelleting in RPMI media. Volume of RPMI added to total gargle volume was very minimal.

Individual sample assay for KSHV. In this assay, samples from an animal model, specifically a mouse, were being used. DNA was extracted from blood cell pellets, spleen cell pellets, and bone marrow cell pellets using the Promega Wizard® SV Genomic DNA Purification System (Table 4) with the protocol for isolating genomic DNA from cells using a microcentrifuge. DNA was eluted in 150µL.

High-throughput EBV screen. LCLs are grown up in 384-well plates and variably treated with drug. Identical wells are then combined and transferred to a 96-well plate (GeneMate 96-well semi-skirted PCR plate, ISC BioExpress). Cells are pelleted by spinning in a centrifuge. Supernatant is removed and 100µL of lysis buffer is added. Lysis buffer consists of 10mM Tris HCl, pH=8, 2.5mM $MgCl_2$, 1% Igepal, 1% Tween, all in sterile water, with 1mg/mL of proteinase K added right before use. Once the sample plate is prepared, the plate is sealed and incubated at 55ºC for 45 minutes in order to activate the proteinase K. The plate is then transferred to a 96-well Eppendorf Mastercycler gradient PCR machine and incubated at 99ºC for 15 minutes. The plate is

then spun down for approximately 6 seconds in a centrifuge to ensure lack of air bubbles, and to minimize spillage.

High-throughput KSHV screen. Cells were grown up in 96-well tissue-culture plates in supplemented RPMI 1640 media. When cells reached a concentration of approximately 8 x 10^5 cells/mL, 100µL was transferred to a 96-well plate (GeneMate 96-well semi-skirted PCR plate, ISC BioExpress). The plate was sealed and spun in a centrifuge at 250 x g for 5 minutes. Once the cells pelleted, 80µL of media was removed, and 100µL of water added to each well. Cells were pipetted up and down to ensure resuspension of the pellet. The plate was then incubated at 99ºC for 15 minutes in the MJ Research Opticon 2 Continuous Fluorescence Detection System.

PCR product cloning and sequences. In order to verify that the product being amplified in the QPCR reactions was indeed the desired one, PCR product was cloned and sequenced. PCR product was gel-extracted and purified using the Millipore Ultrafree DA Kit. The product was further purified using the Qiagen QIAquick PCR Purification Kit. Insert was prepared with the END-IT™ DNA End-Repair Kit, and vector was dephosphorylated using SAP, and both were cut by EcoRV in preparation for ligation. The insert was then ligated into a pBluescript II KS (+) vector (Stratagene, La Jolla, CA), using T4 DNA Ligase (NEB, Ipswich, MA). The subsequent ligation was heat-shock transformed into DH5α competent cells, then plated with IPTG and X-gal. By using the X-gal color assay, we were able to only select the white colonies as those that may contain the insert. Twelve colonies were picked and left to grow up. A mini-prep of these cultures was conducted using the Fermentas GeneJET™ Plasmid Miniprep Kit. Resulting DNA was run out on a 1% agarose gel and then sequenced by Eton

Biosciences, Inc. (San Diego, CA). We cloned and sequenced a LANA78 product, and two of the resulting clones were positive and once sequenced, matched the BLAST database for the LANA78 region. The agarose gel showing the positive clones can be seen in Figure 1.

Statistical analysis and computer software. All statistical analysis and data output was calculated using Microsoft® Excel 2004 software (Microsoft). Program used to run Opticon 2 Continuous Fluorescence Detection System is Opticon Monitor v. 2.02.24 (MJ Research, Inc.). Lightcycler® 480 was run with Lightcycler® 480 software release 1.3.0.0705 (Roche). Program used to run MWG RoboGo! pipetting robot is Robo Manager NT v. 4.0.10 (MWG Biotech, Inc.)

Table 1. List of primer sequences.

U6	*F*	5'-CTCGCTTCGGCAGCACA-3'
	R	5'-AACGCTTCACGAATTTGCGT-3'
LANA78	*F*	5'-GGAAGAGCCCATAATCTTGC-3'
	R	5'-GCCTCATACGAACTCCAGGT-3'
K6	*F*	5'-CGCCTAATAGCTGCTGCTACGG-3'
	R	5'-TGCATCAGCTGCCTAACCCAG-3'
ERV3	*F*	5'-CATGGGAAGCAAGGGAACTAATG-3'
	R	5'-CCCAGCGAGCAATACAGAATTT-3'
Hel	*F*	5'-CCTCTACACCGCCGTCAC-3'
	R	5'-ACAAAGCCCAGGATGAACTC-3'
EBNA3c	*F*	5'-AAGGTGCATTTACCCCACTG-3'
	R	5'-AGCAGTAGCTTGGGAACACC-3'
Alu	*F*	5'-GACCATCCCGGCTAAAACG-3'
	R	5'-CGGGTTCACGCCATTCTC-3'

Table 2. Mapping of synthetic oligonucleotide targets and primers onto BLAST sequences. Primers underlined in red, oligonucleotide components in bold.

Gene	Accession #	Sequence
U6	NR_004394	GTG**CTCGCTTCGGCAGCACATATAC**TAAAATTGGAA CGATACAGAGAAGATTAGCATGGCCCCTGCGCAAGG **ATGACACGCAAATTCGTGAAGCGTT**CCATATTTT
LANA78	AF192756	……AGACGGT**GGAAGAGCCCATAATCTTGCACGGG TCGTCATCC**GAGGACGAAATGGAAGTGGATTACCCT GTTGTTAGCACACATGAACAAATTGCCAGTAGCCCAC CAGGAGATAATACACCAGACGATGACCCACAACCTG GCCCATCTCGCGAATACCGCTATGTACTCAGAACATC ACCACCC**CACAGACCTGGAGTTCGTATGAGGCGC**G TTCCA……
K6	U74585	……ACTCGTGTCGTACA**CGCCTAATAGCTGCTGCTA CGGGTTCCA**GCAGCACCCACCGCCCGTCCAAATTCT AAAAGAGTGGTATCCCACGTCCCCAGCGTGCCCAAAA CCCGGAGTTATTTTGCTGACCAAGCGAGGGCGTCAGA TCTGCGCAGACCCTTCCA**AAAACTGGGTTAGGCAGC TGATGCA**GCGGC……
ERV3	NM_001007253	……ACCAATGGC**CATGGGAAGCAAGGGAACTAATG CCCCAA**GATAATTTCACACTAACCGCCTCTTCCCTCG AACCTGCACCATCAAGTCAGAGCATCTGGTTCTTAAA AACCTCCATTA**TTGGAAAATTCTGTATTGCTCGCTG GG**GAAAGGCCT ……
Hel	V01555	……GTTGGTGCC**ACAAAGCCCAGGATGAACTCGTCT GCATAAGCCC**AGGTCAGTCCTAGGTCAGCGGCCGCG TGTAGGAGAACCC**GGGTGACGGCGGTGTAGAGG**CC CCCGAG……
EBNA3c	V01555	……TACAGTC**AAGGTGCATTTACCCCACTGGACATT** AATGCCACCACGCCAAAAAGGCCTCGAGTAGAAGAA AGTTCTCACGGACCT**GCCCGGTGTTCCCAAGCTACT GCT**AGGACGCG……

Table 3. Oligonucleotide target dilution series. From initial stock concentration of 6 x 10^6 molecules/µL, 8 five-fold dilutions made. 5µL used in the QPCR reaction.

molecules/µL	copy number
1200000	6000000
240000	1200000
48000	240000
9600	48000
1920	9600
384	1920
76.8	384
15.36	76.8

Table 4. Theoretical amplification introduced by Promega Wizard® SV Genomic DNA Purification System (assumes 1 copy of EBV/KSHV genome/µL input or 10^3 copies/mL)

Step:	**Promega**
input volume	600 µL
elution volume	100 µL
amplification factor	6x
copies/µL	6
Standard QPCR	
maximal replicates	11+1
input volume	8 µL
final volume	30 µL
amplification factor	0.27x
copies/well	48

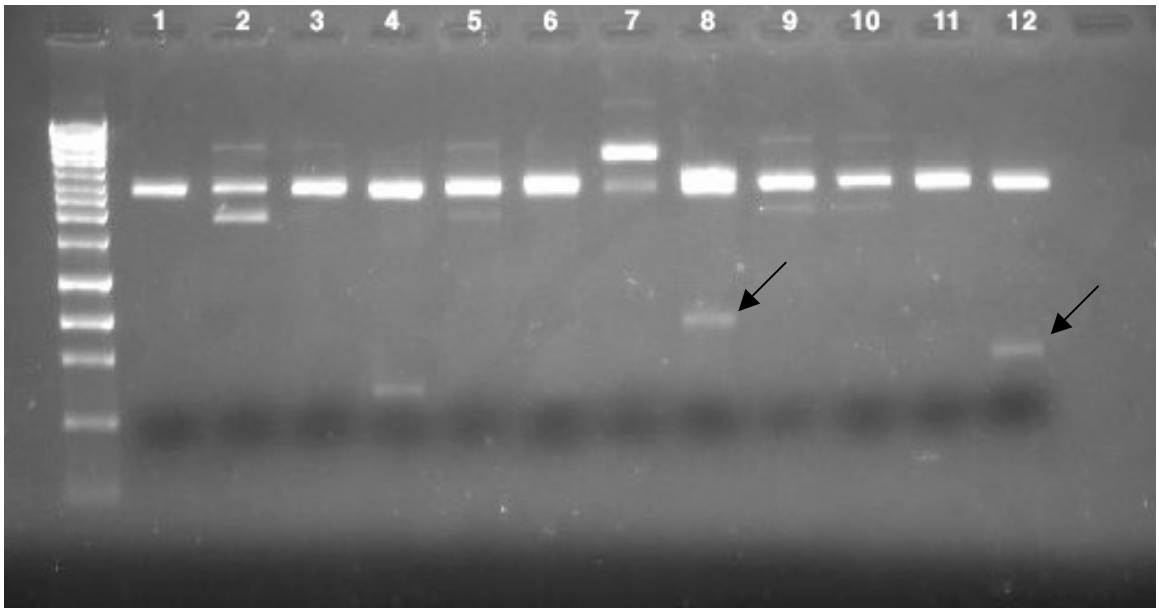


Figure 1. 1% Agarose gel of cloned LANA78 PCR products. Lanes 8 and 12 yielded clones that upon sequencing were found to match the BLAST alignment for LANA78 (See Table 2). Lane 8 represents a head-to-head trimer of LANA78, Lane 12 represents a head-to-head dimer of LANA78, and Lane 4 represents an irrelevant product.

III. RESULTS

Differences between target DNA and positive control established. In order to assure that there is no cross-contamination during robot pipetting or sample preparation, a precaution was built into the assays. As mentioned previously, for each primer set there is a synthetic oligonucleotide target that can behave as a positive control for the primers (Table 2). These oligonucleotides were designed so that their sequence is significantly shorter than that of the PCR product from sample DNA, i.e. they are shorter than the full-length product. This allows us to perform a qualitative control on our results by visualizing the PCR products with agarose gel electrophoresis. We expect the synthetic oligonucleotide to run more quickly and appear further down the gel than the full-length amplicon. It is clear that there is a detectable difference between the synthetic oligonucleotide target used for the standard curve for both the individual sample assay for KSHV (Figure 2) and the individual sample assay for EBV (Figures 3A and B). We can also see in Figs. 2 and 3 that it is established that there is no cross-contamination during any point of the assay – we do not see the shorter amplicon from the standard in the wells of the sample.

Linearity. In order to validate an assay, we need to establish that the standard curve of the assay approximates a straight line (9). The linear standard curve also defines the linear range of the assay. If values from various samples rise above or below the linear range, we cannot make conclusions about the viral load contained in that sample

from the standard curve. We can see that for both the EBV assay (Figure 4) and the KSHV assay (Figure 5), the assay is linear as reflected by the R^2 value.

For the EBV assay, the ERV, EBNA3c and Hel primers have R^2 values of 0.984, 0.9905, and 0.9838 respectively using n=8 dilution points.

For the KSHV assay, the ERV, K6 and LANA78 primers have R^2 values of 0.9958, 0.9936, and 0.9863 respectively using n=8 dilution points.

Precision. In order for the assays to be functional, a certain level of precision needs to be obtained for each primer set used. Precision requires that the assay achieve similar viral quantification values regardless of external variables for same samples. Precision is quantified by determining the change in viral load that is statistically significant. DHHS guidelines state that in a valid assay, a viral load change of 3-fold (0.5 log 10) is the minimum change that is considered significant (9). Here we will show that we have achieved this for the EBV and the KSHV assay. We used 2-fold increases in cell number ranging from 1000 to 32000 cells. The data combine a variation of sample preparation and QPCR since the number of cells indicated for each sample represents to number of cells input into the DNA purification system.

For the EBV assay, we have attained a high level of precision. From Figure 6, we can see that for the three primers – ERV (Fig. 6A), EBNA3c (Fig. 6B) and Hel (Fig. 6C), we are able to detect a 2-fold difference with statistical significance – more precise than the DHHS requirement of 3-fold detectable difference. This difference was detectable through the entire range of target cell count that we tested – 1000 cells to 32000 cells. This validates the use of these three primers in the EBV assay as precise. Regression analysis for this data can be seen in Table 5.

For the KSHV assay, we see in Figure 7A that the ERV primer can accurately detect 2-fold differences between 1000 and 16000 cells with statistical significance. Once the cell count, and therefore amount of virus being measured, reaches this threshold, we cannot ascertain the 2-fold difference with statistical significance. Therefore to a cell count of 16000 we have achieved a precision of a 2-fold change – better than the DHHS minimum of 3-fold. Figure 7B shows the data for the K6 primer. We see that this is far less able to accurately detect the differences in target. We cannot accurately detect 2-fold differences for K6, but a 4-fold difference in target is statistically significant. This is greater than the 3-fold difference prescribed by DHHS, so we cannot use the K6 primer in a validated assay. However, in Figure 7C we see that for the LANA78 primer, we have achieved a precise result. A two-fold difference in target cell number can be detected with statistical significance across the whole range tested, from 1000 to 32000 cells, exceeding DHHS standards. Regression analysis for this data can be seen in Table 6.

Dynamic Range. The data from Figs. 6 and 7 not only demonstrates the precision of the assay, it quantifies the dynamic range of the assay. Dynamic range is defined by the quantitative range over which the assay can reliably report results (9). For both the EBV and KSHV assays, we tested a range of 1000 to 32000 EBV+ or KSHV+ cells with 2-fold differences.

For the EBV assay, all three primers report statistically significant differences between each of the 2-fold differences, allowing us to say these differences are precise. Therefore the dynamic range of the EBV assay is 1000 to 32000 cells. However, the

upper limit of this assay is still unexplored, and it is possible that the dynamic range is in fact greater than 32000 cells.

For the KSHV assay, the LANA78 primer was able to detect the full range tested – from 1000 cells to 32000 cells. ERV was able to detect up to 16000. However, K6 was unable to achieve this level of precision. Because LANA78 and K6 are both measures for KSHV copy number, we can change the assay from using three primers to two – only ERV and LANA78, to achieve a dynamic range of 1000 to 16000 cells.

Sensitivity. The sensitivity of an assay is measured by the Limit of Detection (LoD), which is the minimal number of cells, i.e. the lowest viral load level, that are distinguishable from the negative control (NTC) (9).

In Figure 8, we calculated the sensitivity of the EBV assay for the three primers ERV (Fig. 8A), EBNA (Fig. 8B) and Hel (Fig. 8C). We can see for the ERV primer, that we could not distinguish between 100 cells and the NTC, however there was a statistically significant difference between 1000 cells and the NTC, establishing 1000 cells as our LoD for the ERV primer (Fig. 8A). The EBNA primer's results are more complex. We see that for 100 cells, we have two different results. For the 100a target cell sample, we see that there is a statistical significance from the NTC, however for the 100b target cell sample, we cannot distinguish between it and the NTC. Because one of the supposedly identical samples was not different from the negative control, we cannot use 100 cells as our LoD for the EBNA primer, however 1000 cells is clearly statistically distinguishable from the NTC, therefore our EBNA LoD is also 1000 cells (Fig. 8B). The Hel primer yields similar results. There is no statistical significance between 100 cells and the NTC, but the difference between 1000 cells and the NTC is well established

(Fig. 8C). Therefore we can conclude that for the entire EBV assay, our LoD is 1000 cells.

For the KSHV assay, we calculated the sensitivity of the three primers ERV, K6 and LANA78 (Figure 9). For ERV, we see that both 100a and 100b cell samples show a statistically significant difference when compared to the NTC, therefore we can confidently say that the LoD for ERV in this assay is 100 cells (Fig. 9A). However, for the K6 primer, we see that the assay is only sensitive to 10000 cells, and anything less than that does not differ statistically from the NTC, giving this primer an LoD of 10000 cells (Fig. 9B). For LANA78 we have a result similar to ERV in that the LoD is 100 cells (Fig. 9C). We see that for both 100a and 100b target cell samples, they are vastly statistically significantly different from the NTC.

Specificity. The specificity of an assay is determined by its ability to only assess our target, i.e. if the assay is specific, it will have a low rate of false-positives (9). With our assays, to look at specificity we can examine the NTCs for both the EBV and KSHV assays. We can confirm that our assay is specific in two ways – qualitative and quantitative. By examining Fig. 2 for the KSHV assay and Fig. 3 for the EBV assay, we can qualitatively see from the agarose gel electrophoresis that the NTC lanes are empty – no product was amplified, whereas product was amplified in both the sample and positive control lanes. Visually, we can confirm no false positives. However it is important to ascertain quantitatively that there were no false positives. We can see by looking at Fig. 8 for the EBV assay, that the values for the NTC were zero for the ERV, EBNA and Hel primers – meaning that no product was seen even by quantitative analysis in the whole assay. This is confirmed not once, but twice for each primer set as two different NTCs

were run. In Fig. 9 we see the same result for all the primers – ERV, K6, and LANA78. None of the NTCs yielded any product. This establishes that our assays have a high specificity.

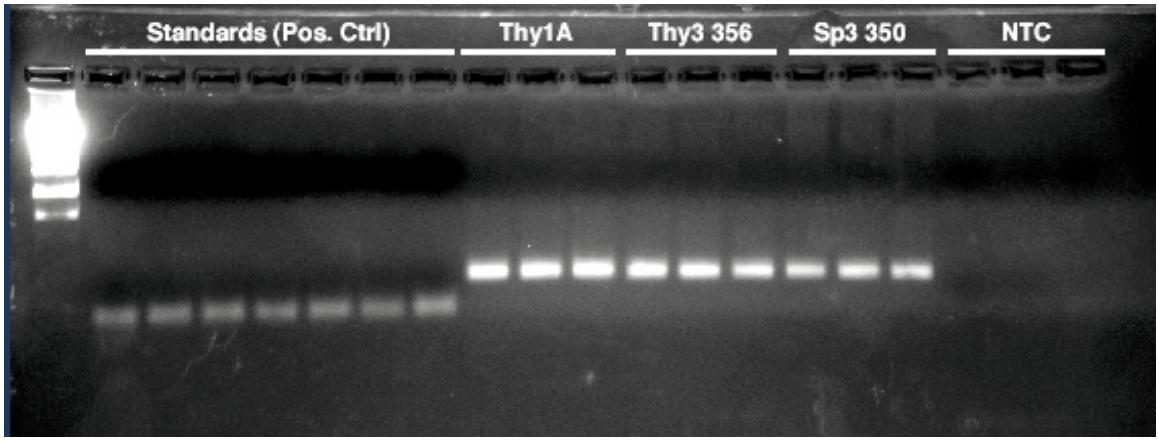
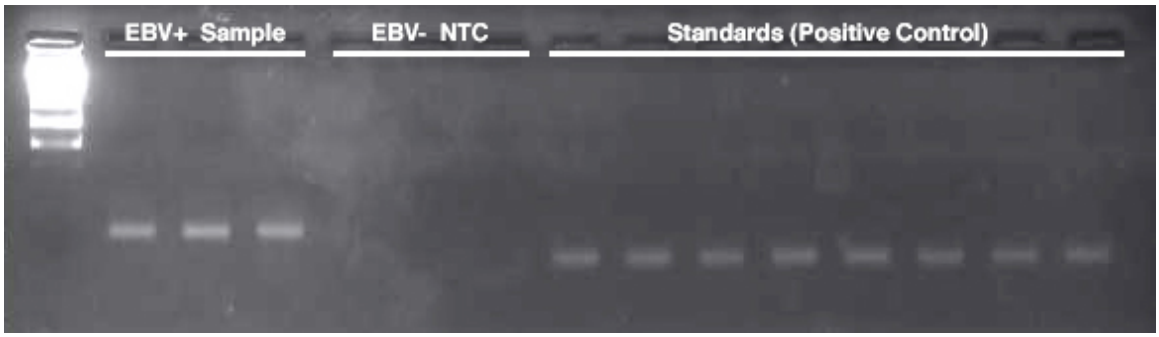


Figure 2. Establishment of differences between synthetic oligonucleotide positive control and full-length sample amplicon in the individual sample KSHV assay. Samples Thy1A and Thy3 356 are both thymus samples from the mouse model. Sp3 350 is a spleen sample. Shows the gel resulting from the samples primed by LANA78. Positive control lanes clearly run longer than sample lanes, and there is clearly no cross-contamination as the NTC does not show either the faster or slower band.

A



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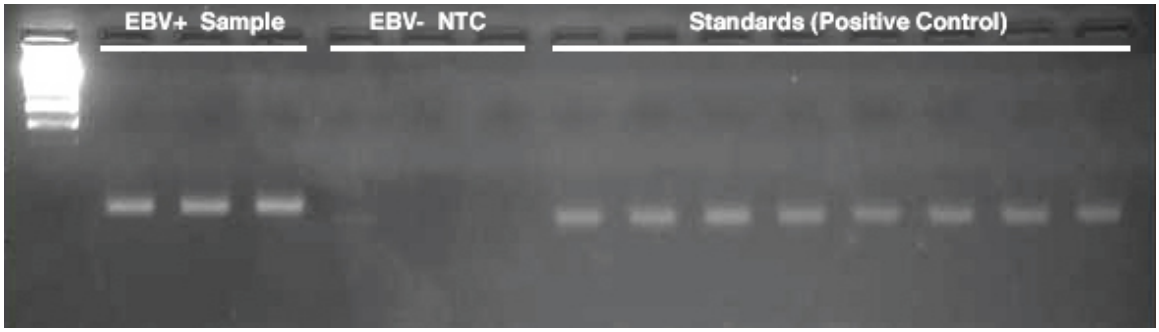
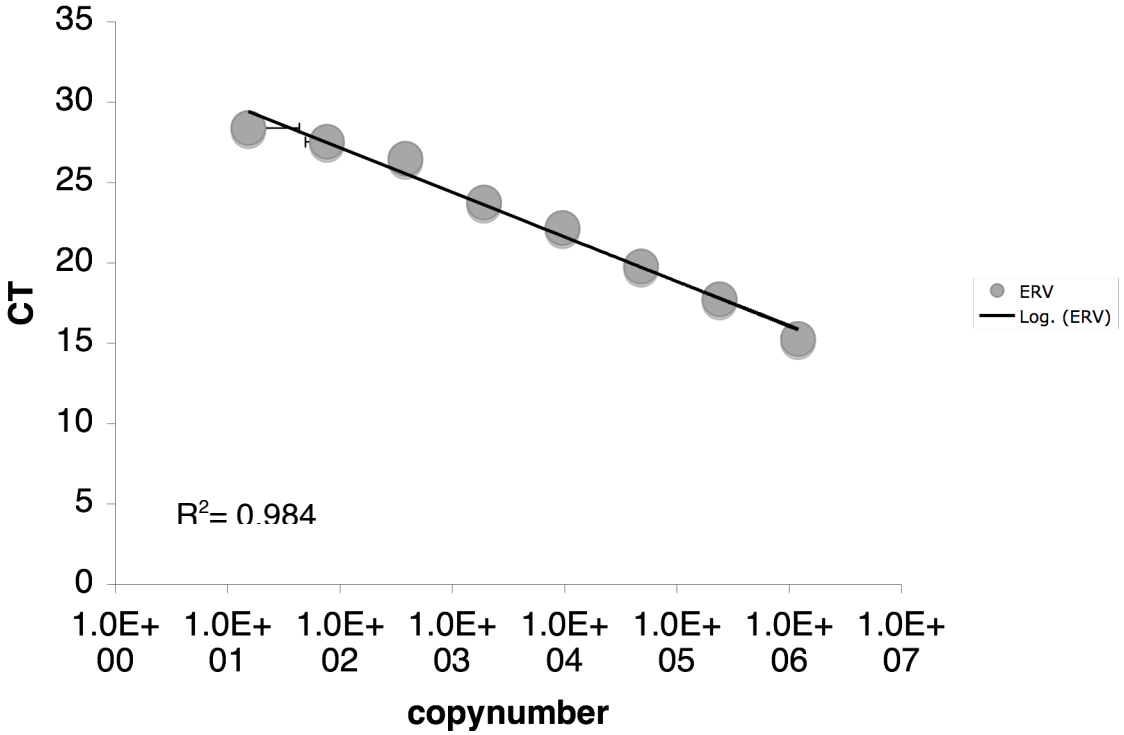


Figure 3. Establishment of differences between synthetic oligonucleotide positive control and full-length sample amplicon in the individual sample EBV assay. EBV + sample in this case was a subject sample (DNA from gargle pellet) supplemented with 10^3 B95-8 cells. (A) Shows the gel resulting from the samples primed by ERV. Positive control lanes clearly run longer than EBV+ sample lanes, and there is clearly no cross-contamination as the NTC does not show either the faster or slower band. (B) Same as Figure 3A, except primed by EBNA3c.

A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25
20
15
10
5
0
CT
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1.0E+01
1.0E+02
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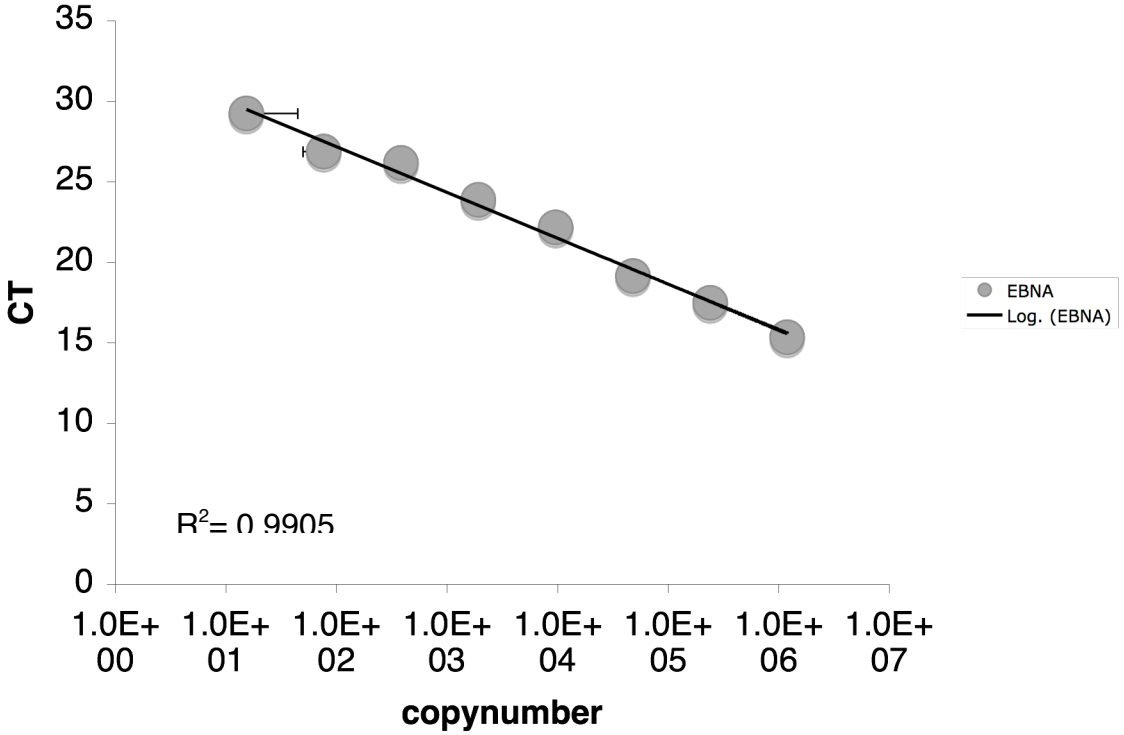
1.0E+06
1.0E+07
copynumber
ERV
Log. (ERV)

B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1.0E+07
copynumber
EBNA
Log. (EBNA)

C

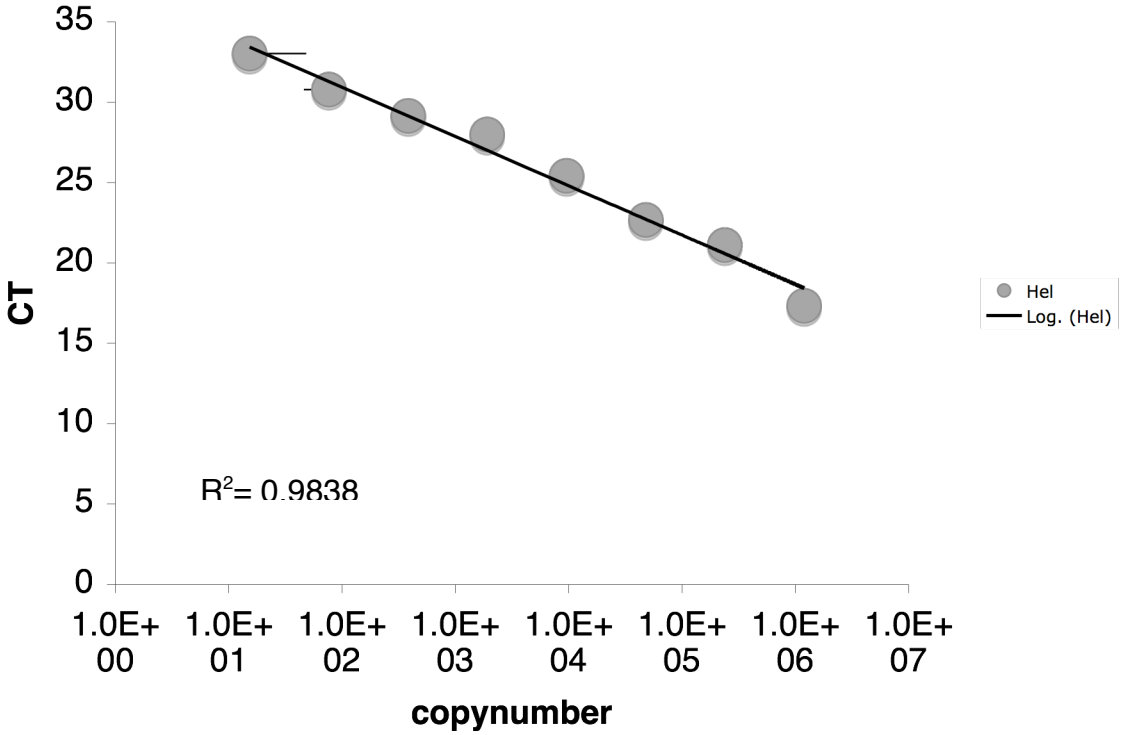
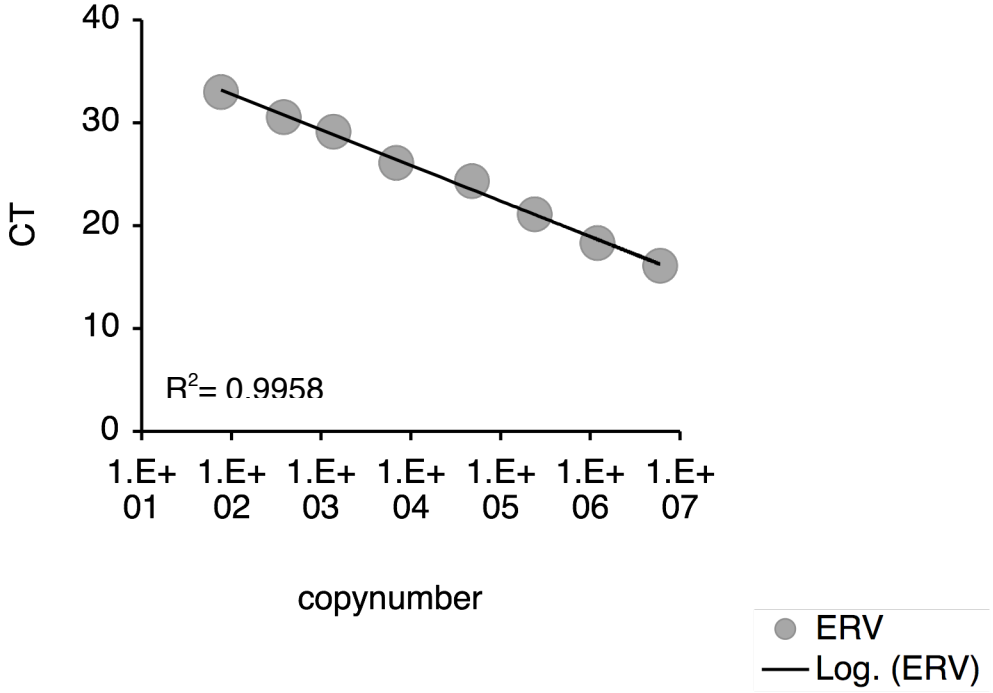


Figure 4. Establishing the linearity of the assay for EBV. Using the synthetic oligonucleotide positive controls, we established a linear standard curve. (A) For the ERV primer, the calculated R^2 value is 0.984. (B) For the EBNA primer, the calculated R^2 value is 0.9905. (C) For the Hel primer, the calculated R^2 value is 0.9838. These data establish the linearity of our assay in the range of our standards.

A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CT
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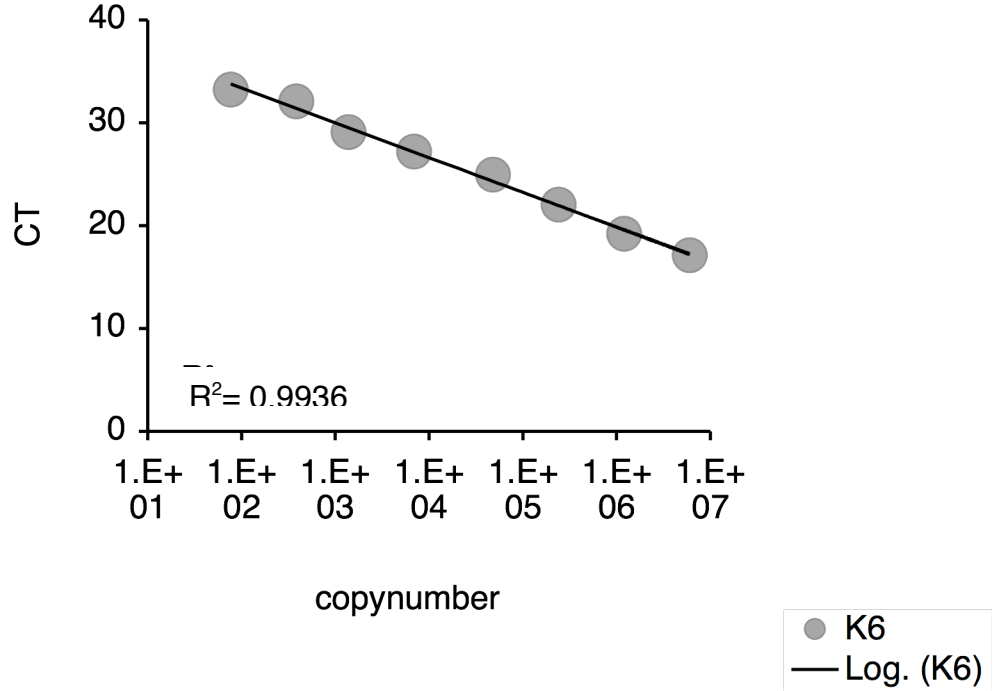
1.E+07
copynumber
ERV
Log. (ERV)

B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30
20
10
0
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copynumber
K6
Log. (K6)

C

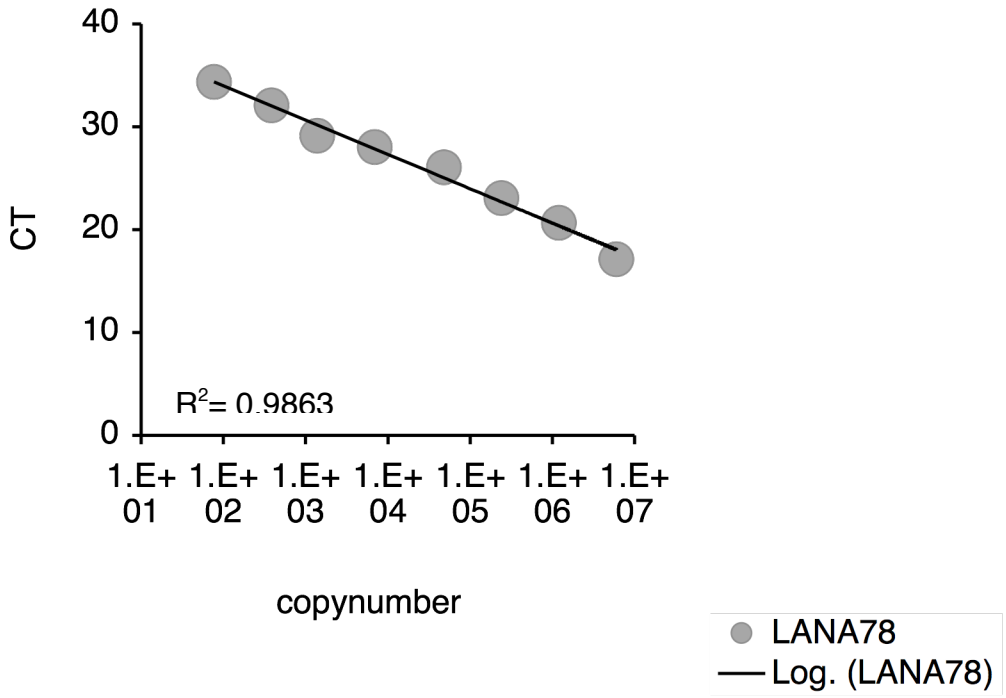
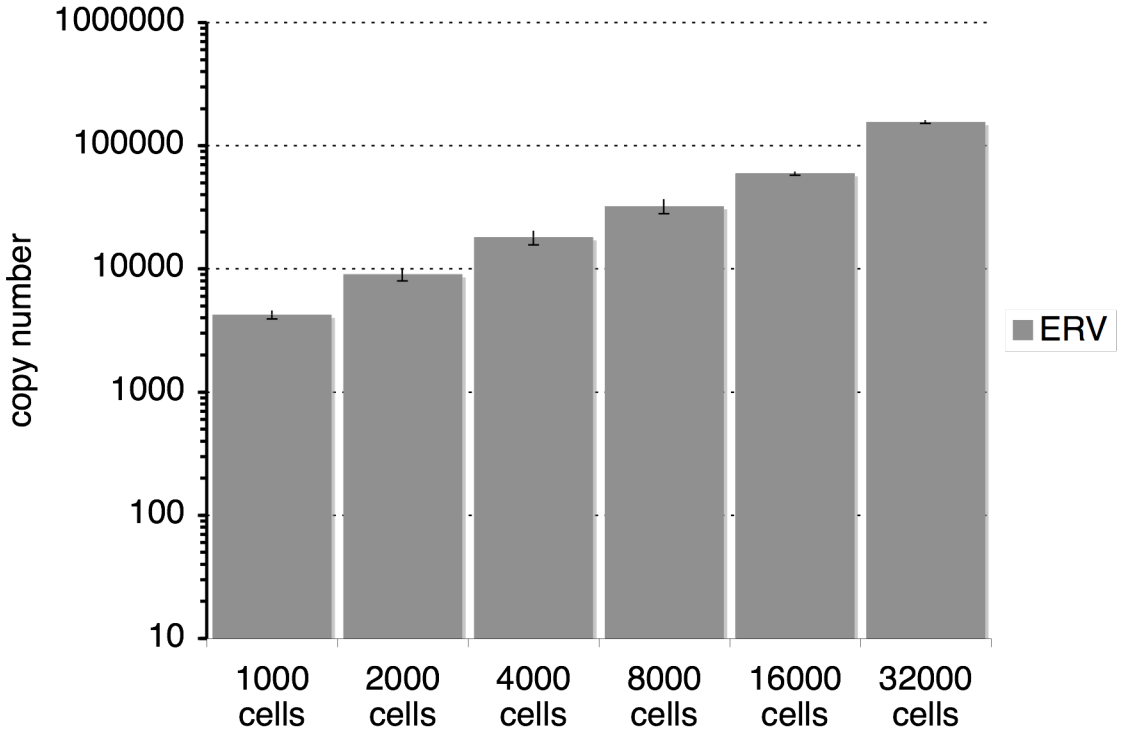


Figure 5. Establishing the linearity of the assay for KSHV. Using the synthetic oligonucleotide positive controls, we established a linear standard curve. (A) For the ERV primer, the calculated R^2 value is 0.9958. (B) For the K6 primer, the calculated R^2 value is 0.9936. (C) For the LANA78 primer, the calculated R^2 value is 0.9863. These data establish the linearity of our assay in the range of our standards.

A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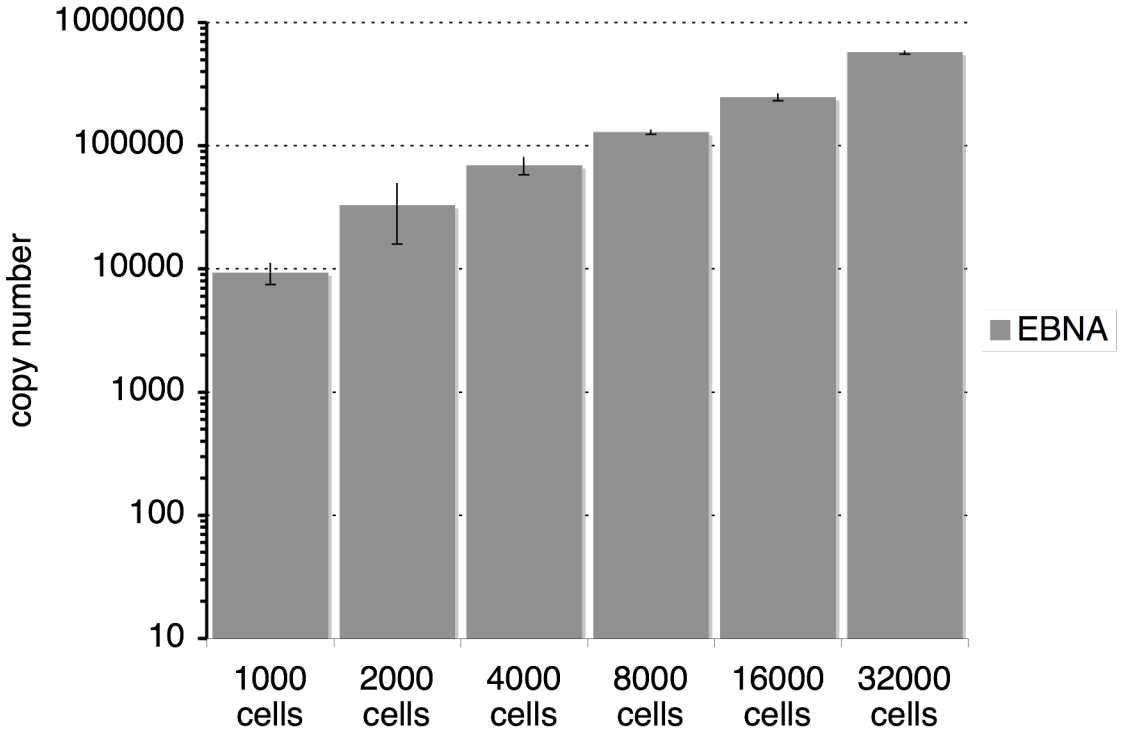
100000
10000
1000
100
10
copy number
1000 cells
2000 cells
4000 cells
8000 cells
16000 cells
32000 cells
ERV

B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copy number
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EBNA

C

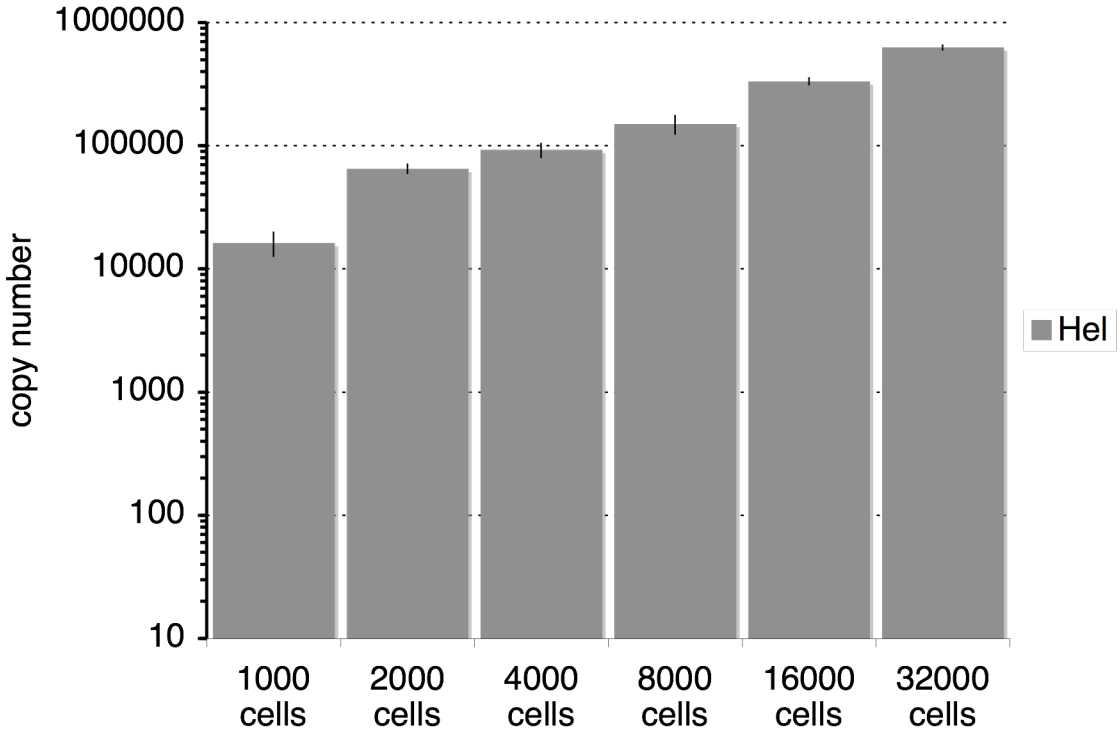
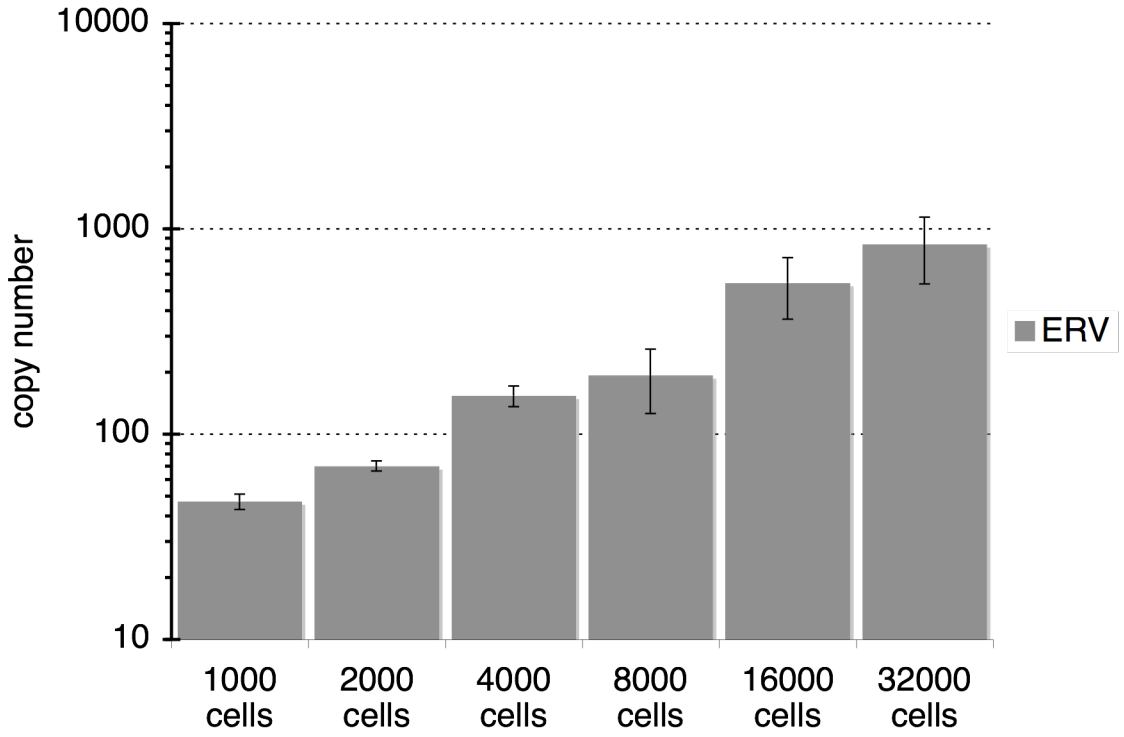


Figure 6. Quantification of the level of precision in the EBV assay. In this experiment, we quantified the viral load of samples with a 2-fold difference in EBV+ cell count (in this case BC1 cells), run in triplicate. Our range was from 1000 cells to 32000 cells. We ascertained whether the assay was capable of detecting this 2-fold difference using the primers (A) ERV (B) EBNA and (C) Hel.

Table 5. Regression analysis for the quantification of precision (Fig. 6) in the EBV assay.

	R^2	Slope
ERV	0.9785	4.9498
EBNA	0.9891	17.471
Hel	0.9755	18.889

A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10
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ERV
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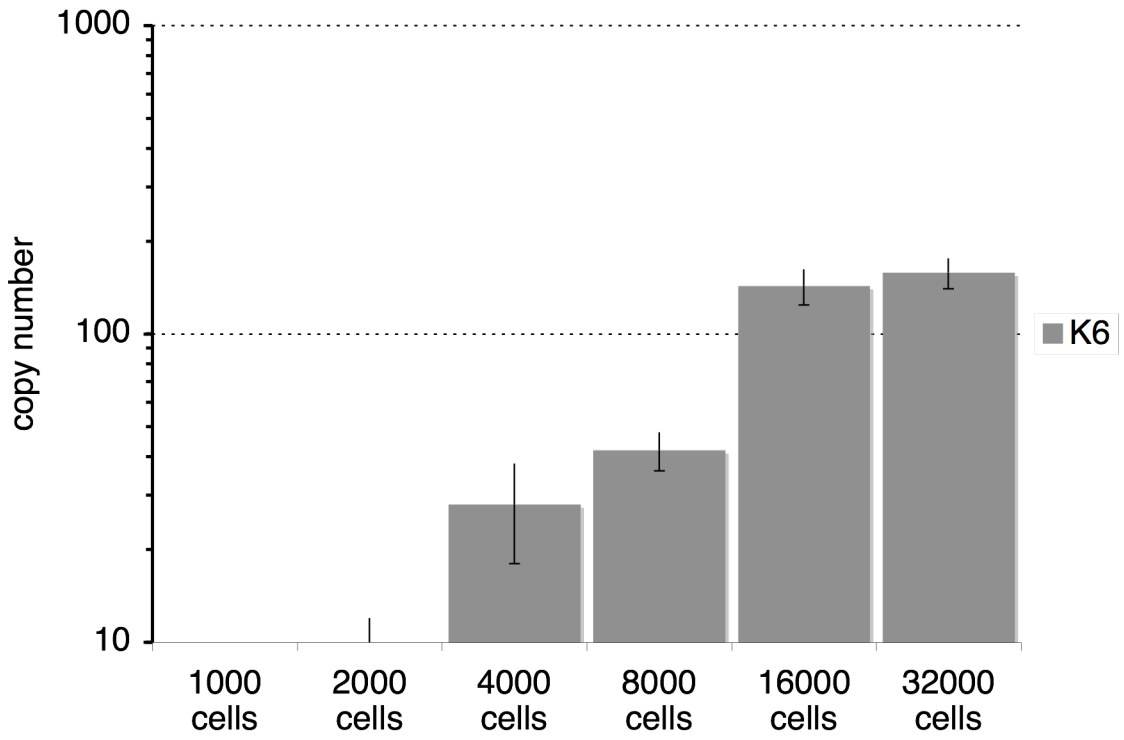
16000 cells
32000 cells

B


1000
100
10
copy number
K6
1000 cells
2000 cells
4000 cells
8000 cells
16000 cells
32000 cells

C

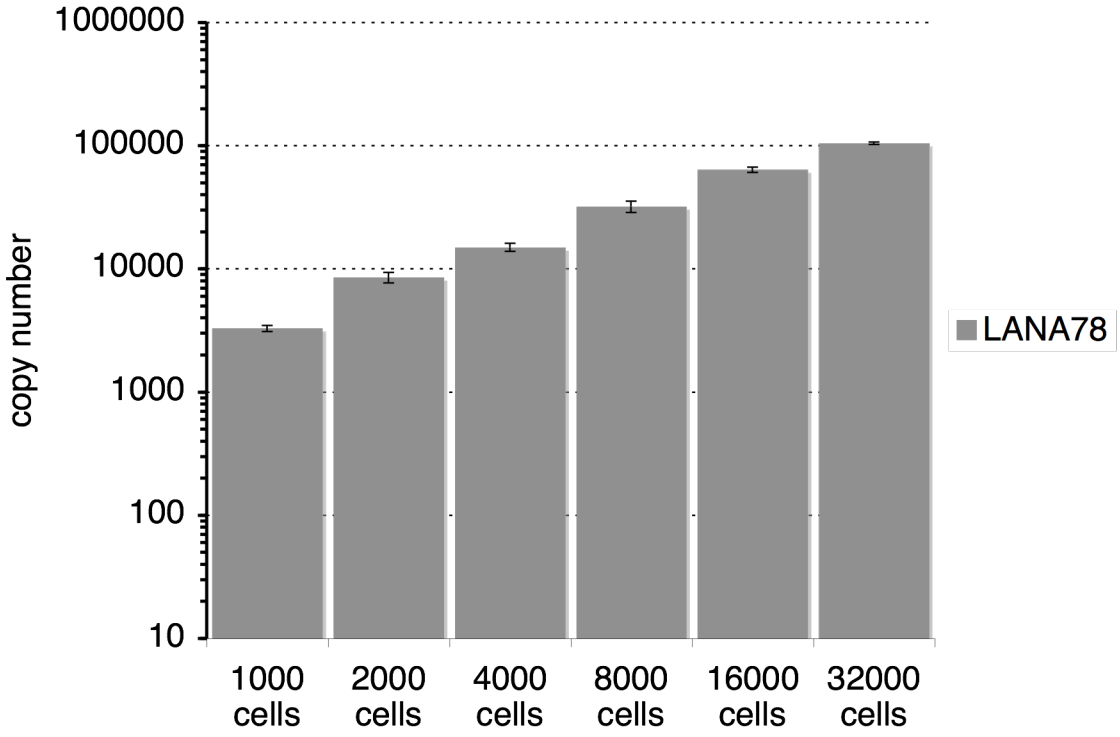
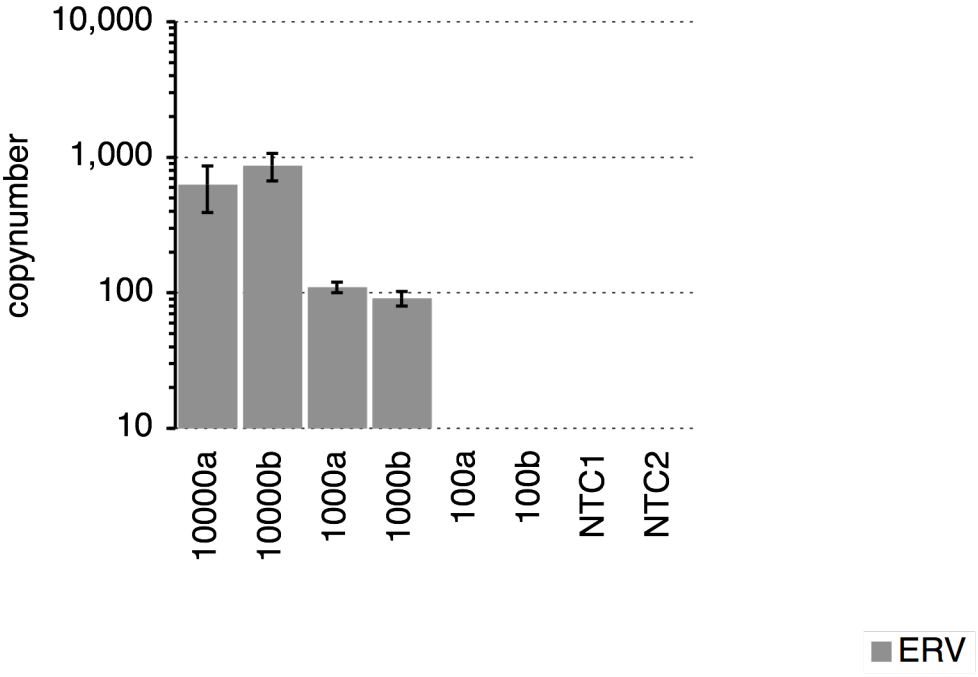


Figure 7. Quantification of the level of precision in the KSHV assay. In this experiment, we quantified the viral load of samples with a 2-fold difference in KSHV+ cell count (in this case BCBL-1 cells), run in triplicate. Our range was from 1000 cells to 32000 cells. We ascertained whether the assay was capable of detecting this 2-fold difference using the primers (A) ERV (B) K6 and (C) LANA78.

Table 6. Regression analysis for the quantification of precision (Fig. 7) in the KSHV assay.

	R^2	Slope
ERV	0.6327	0.0254
K6	0.8045	0.0053
LANA78	0.9773	3.2399

A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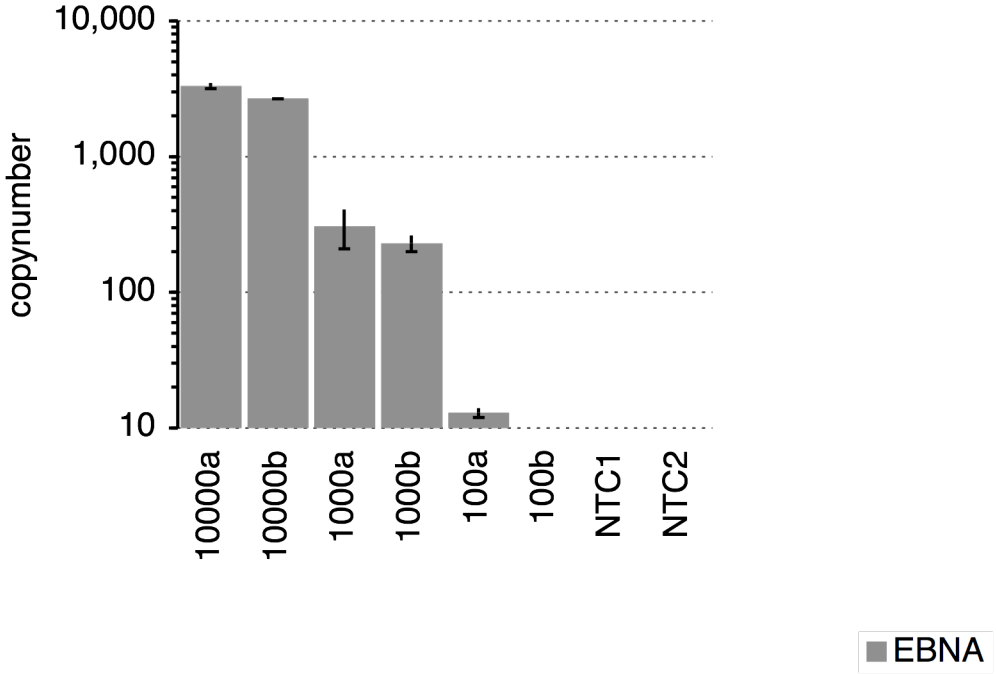
NTC2
ERV

B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NTC1
NTC2
EBNA

C

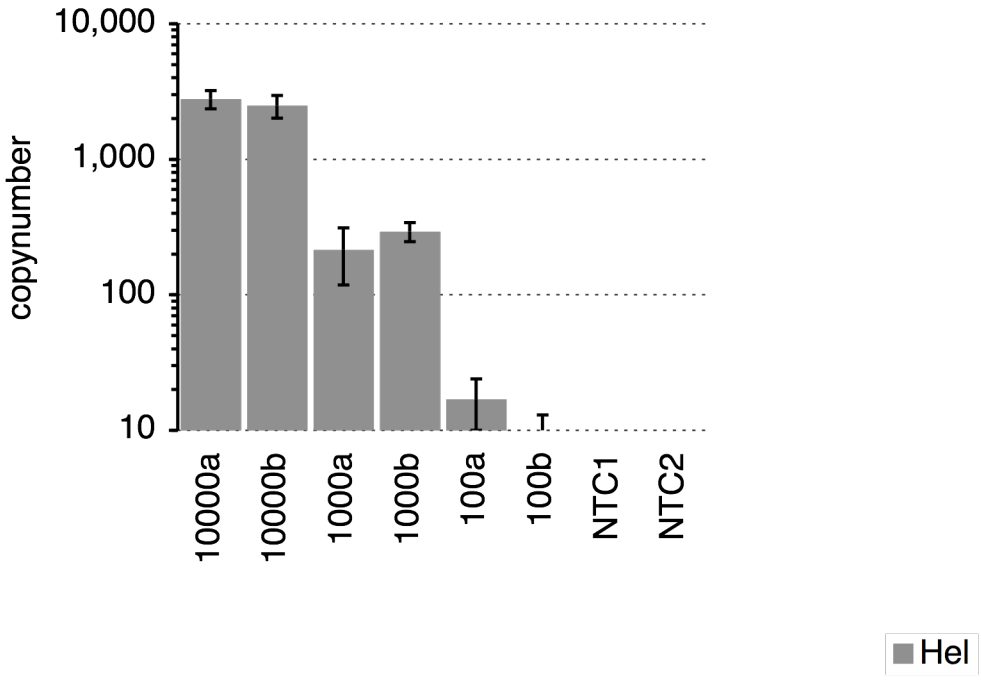
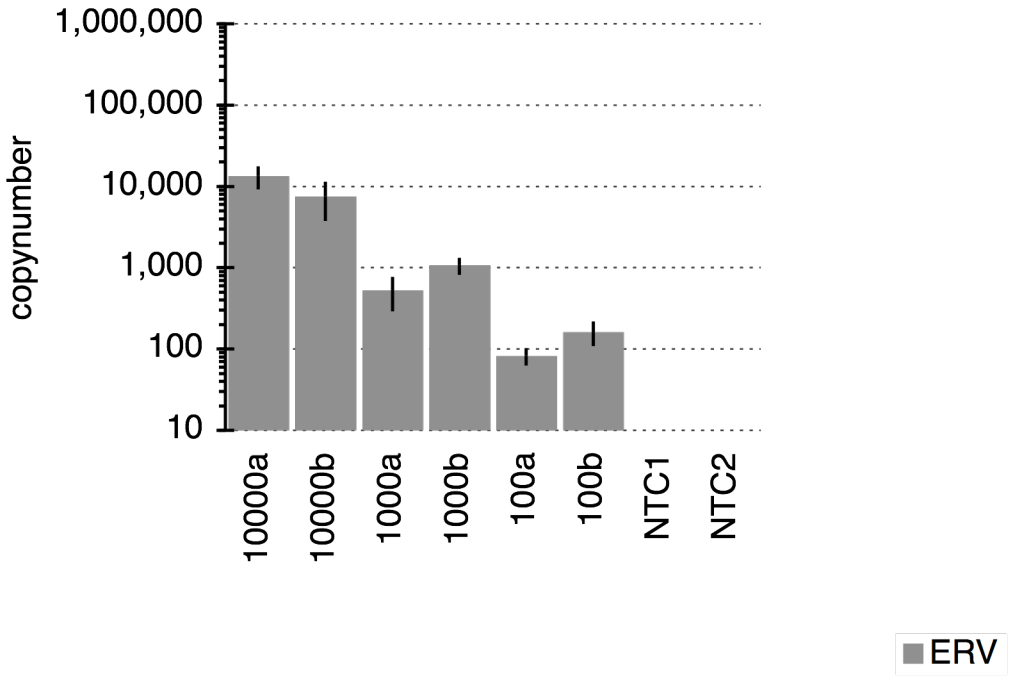


Figure 8. Quantifying sensitivity for the EBV assay. The copy number found in samples for 100000, 1000, 100 and no EBV+ BC1 cells as assayed by the primers (A) ERV, (B) EBNA and (C) Hel. Each sample was made in duplicate, i.e. 10000a, 10000b, and each was run in triplicate.

A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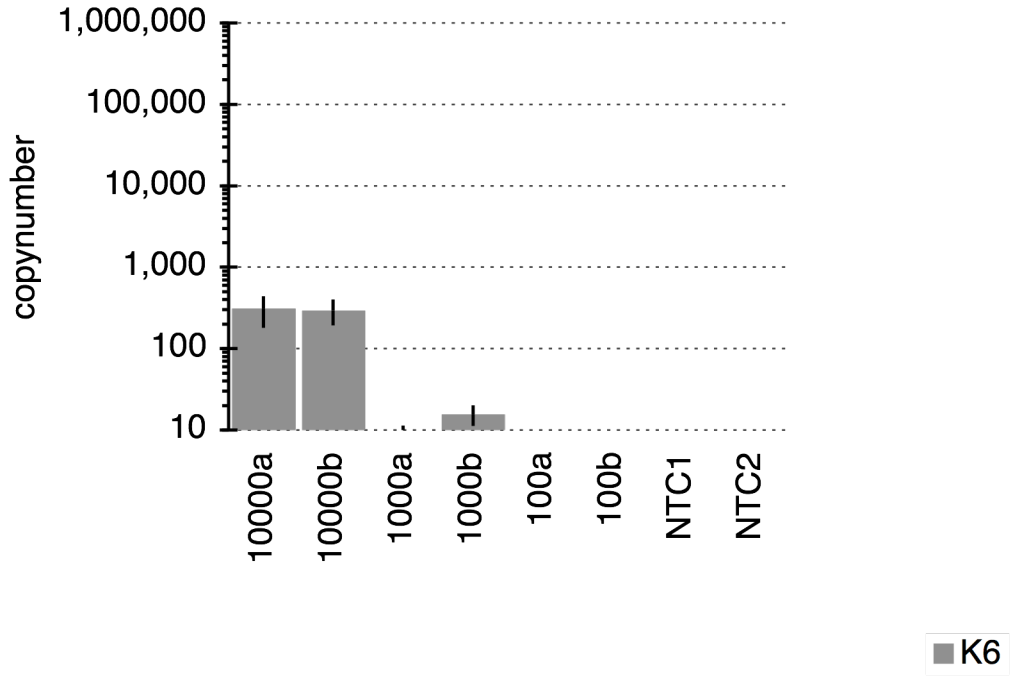
NTC1
NTC2
ERV

B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NTC2
K6

C

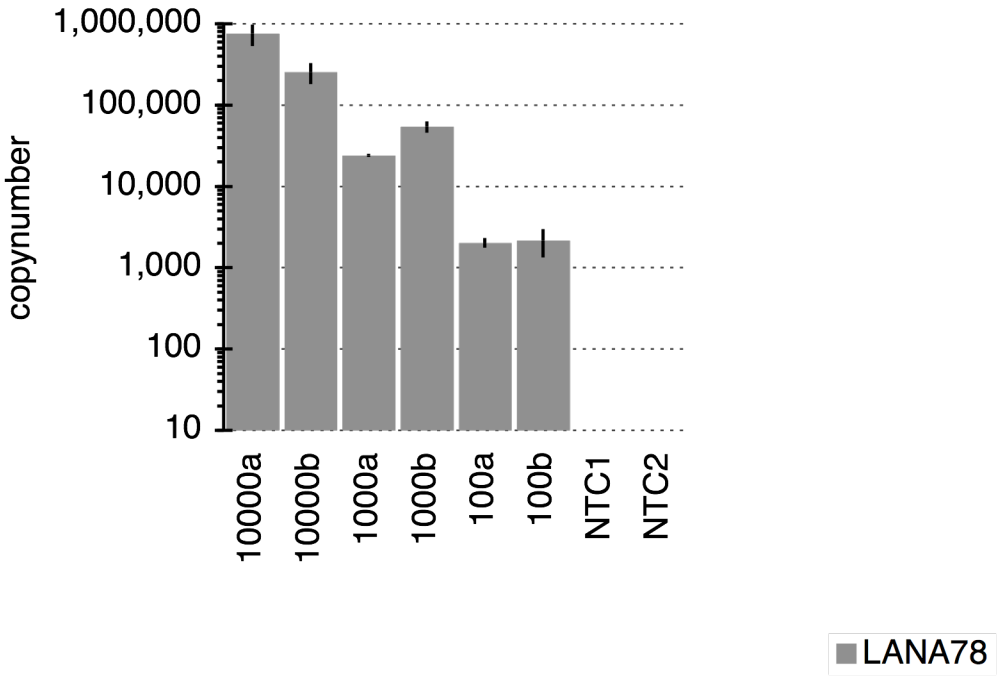

Figure 9. Quantifying sensitivity for the KSHV assay. The copy number found in samples for 100000, 1000, 100 and no KSHV+ BCBL-1 cells as assayed by the primers (A) ERV, (B) K6 and (C) LANA78. Each sample was made in duplicate, i.e. 10000a, 10000b, and each was run in triplicate.

IV. DISCUSSION

Establishing a validated assay. In this study, we established assays for viral load of KSHV and EBV. Constructing easy-to-use and low-cost assays that are quantitative such as these to use as tools in the laboratory is very useful, not only for the experiment they were designed for, but because they can be applied to a broad range of projects. In these assays, various kinds of samples are used, for purposes ranging from basic research to medical diagnostics.

We validated the assays using the parameters of linearity, precision, dynamic range, sensitivity, and specificity. For the EBV assay, we validated the primers ERV3, EBNA3c and Hel, ERV3 primes for a human endogenous retroviral sequence, and is therefore a marker for cellular, not viral, load (33). ERV3 is a single-copy gene, and therefore very meaningful to our assay, because data from the ERV3 primer will more accurately reflect the actual viral load. EBNA3c primes for the EBV nuclear antigen 3c, therefore it is a marker for viral load. Hel is also a marker for an open reading frame (ORF) in EBV. For the KSHV assay, we attempted to validate the primers ERV3, K6 and LANA78. K6 is a primer for the K6 ORF of KSHV, making it a viral marker. LANA78 is another viral primer for the latent nuclear antigen of KSHV.

The EBV assay was validated to all of the parameters. The linearity of the assay was established, as the construct standard curves of each primer had R^2 values between .9838 and .9905, meaning that between 98.38% and 99.05% of the data are accounted for by assuming a linear model (Fig. 4). This is a very high number and not only establishes

our standard curve, but shows that the pipetting in our assay is accurate, and we can confidently use this curve to calculate values for our sample data. The EBV assay also held up under scrutiny when we examined it's precision. For all the three primers – ERV3, EBNA3c, and Hel, we are able to, with statistical significance, detect a 2-fold difference (Fig. 6). This is very good, because the DHHS requirement for a validated assay is that a 3-fold difference be the minimum detectable difference. With a 2-fold difference validated for these primers, we can be confident applying in this assay in the dynamic range of 1000 to 32000 cells. We validated this assay on EBV latently infected BC1 cells, since the two envisioned applications are to measure intracellular viral load. These cells carry more than one viral genome copy per cell, but less than ten. An extension of this work will be to use calibrated free EBV virus such as available from Advanced Biotechnologies incorporated (cat#10-115-000). Although this is quite a precise reading, in the future we would want to expand the dynamic range of the assay and test more intervals of target cell number greater than 320000. For all the of the EBV primers we found that the LoD was 1000 cells – any less than this and we could not detect a difference from the NTC with any statistical significance, therefore we can say that 1000 cells is the bottom end of the sensitivity for this assay (Fig.8). This assay has also proven to be highly specific, priming only positive control and sample, yielding no false positive NTCs. We can see this qualitatively by gel electrophoresis in Fig. 3 and quantitatively by QPCR in Fig. 8. From these data, we see that the EBV assay for the primers ERV3, EBNA3c and Hel was validated and is ready to be applied.

The KSHV assay was also successfully validated. The linearity of this assay was also very high, with the R^2 values for our oligonucleotide standards falling between

0.9863 and 0.9958, meaning that between 98.63% and 99.58% of the data fit to a linear model (Fig. 5). The linearity results for both of the assays are highly robust, establishing that both the KSHV and EBV assays have strong linearity within the range of the positive control standard, and any we can be confident about making calculations of copy number, etc. for a result that falls in this range when we look at non-control samples. When we examined the precision of the KSHV assay, it became apparent that the K6 primer should not be used for this purpose. While the ERV3 and LANA78 primers were both able to detect a two-fold difference in target cell, K6 was only able to detect a 4-fold difference (Fig. 7). We also saw that by quantifying the sensitivity of the assay, K6 would not be a good primer to use for sample analysis. While ERV3 and LANA78 were able to detect 100 cells with statistical significance, K6 was only able to detect 10000, which is not sensitive enough for our purposes (Fig. 9). Therefore, if we only use ERV3 and LANA78 in our assay, it would have an LoD of 100 cells. Like the EBV assay, we can also say that the KSHV assay is highly specific, as we do not see false positives qualitatively on a gel (Fig. 2) or quantitatively by QPCR (Fig. 9). From these data, we can say that we have established a validated KSHV assay for the primers ERV3 and LANA78. We validated this assay on KSHV latently infected BCBL-1 cells, since the two envisioned applications are to measure intracellular viral load. These cells carry more than one viral genome copy per cell, but less than twenty. An extension of this work will be to use calibrated free KSHV virus. This will need to be generated in the future and particle counts will need to be obtained by electron microscopy

Limitations. Although from the data both the EBV and KSHV assay appear robust, there are limitations to both assays in that the number of samples tested is low. In

order to further validate the assay, we should look at the parameter of accuracy, and try to repeat the results we found above multiple times with several different runs, different samples, on different days. This would also allow us to establish inter-plate variation, which would quantify our inter-assay accuracy. Broadening the dynamic range of the assay would also further its usefulness to the field. Further testing the precision of the assay with higher 2-fold increases in cell amount would accomplish this.

Assay vs. screen. By validating the EBV and KSHV assays in this study, we are in turn validating the EBV and KSHV screens that have previously been discussed. In order to establish a correlation between fluorescence and copy number, etc., one needs to establish a base line, in this case our standard curve with the synthetic oligonucleotides. Once this standard has been established and shown to be repeatable within a narrow range, one can apply the statistics from this assay to a screen that employs the same methodology. A screen does not include a standard curve, but typically marks results as either '+' or '-', having or not having the gene of interest. In this case, since we are validating an assay with the same techniques, we can do a quantitative screen and show not only which samples we test have the virus, but also we can assess the viral load.

Applications. The assays and screens developed here were each developed for a singular, unique purpose. The individual sample EBV viral load assay was developed as part of the OHARA project to quantify viral load from a gargle sample from a patient – an easy, painless way to diagnose oral levels of virus. The individual sample KSHV viral load assay was developed to test whether or not a novel mouse model for KSHV was indeed a valid one – whether its organs were expressing the virus or not, and if so, to what levels. The high-throughput EBV viral load screen will be used to assess drug

response in LCLs to a large variety of drugs and different cell lines so that those with the greatest response can be selected and studied further. The high-throughput KSHV viral load screen will be used to study mechanisms of loss and/or shedding of the KSHV episome in B-cells.

All of these applications are extremely worthy, but if we step back and look at the collection of assays developed for very different purposes, we can identify that we have developed a package of assays and screens to assess the levels of two different gamma herpesviruses – EBV and KSHV. This package can be useful to the field in many different ways, and can possibly be expanded in the future to include other herpesviruses.

APPENDIX A. MWG RoboGo! parameters for individual sample assays.

TASK: 3 primers for 8 different samples (each combined with 2x SYBR) into 96 well plate at 30 µl total reaction volume		
OPERATION	**PARAMETER**	**SETTING (100 µL SYRINGE)**
General	Prime	4 µl
	Waste (primer ; sample)	2 µl
	Airgap to System	10 µl
	Separation airgap	0 µl
	Transport airgap	2 µl
	Spit airgap before dispense	Enabled
	Pick airgap when changing slot	Disabled
	Spit system airgap at waste	Enabled
Aspiration	Speed	70 µl/s
	Pre-delay, post-delay	200 ms, 500 ms
	Liquid level sensing	Disabled
	Tracking and submerge	Enabled, 15 steps
	Retract speed	1500 steps/s
Dispense	Speed	120 µl/s
	Pre-delay, post-delay	500 ms, 500 ms
	Liquid level sensing	Disabled
	Tracking and dispense height (dry ; wet)	Enabled, 15 steps
	Retract-speed (primer ; sample)	500 steps/s
Wash/ flush	Repeat	4 times
	Flush syringe at waste pos.; wipe	Enabled; Disabled
	Peripump time	1000 ms
	Detergent time	0
	System airgap after flush	0 µl

APPENDIX B. MWG RoboGo! parameters for high-throughput screens.

TASK: 2 primers for 96 different samples (each combined with 2x SYBR) into 384 well plate at 10 µl total reaction volume		
OPERATION	**PARAMETER**	**SETTING (100 µL SYRINGE)**
General	Prime	2 µl
	Waste (primer ; sample)	2 µl
	Airgap to System	2 µl
	Separation airgap	0 µl
	Transport airgap	2 µl
	Spit airgap before dispense	Enabled
	Pick airgap when changing slot	Disabled
	Spit system airgap at waste	Enabled
Aspiration	Speed	100 µl/s
	Pre-delay, post-delay	100 ms, 200 ms
	Liquid level sensing	Enabled
	Tracking and submerge	Enabled, 15 steps
	Retract speed	1000 steps/s
Dispense	Speed	200 µl/s
	Pre-delay, post-delay	100 ms, 500 ms
	Liquid level sensing	Disabled
	Tracking and dispense height (dry ; wet)	Enabled, 15 steps
	Retract-speed (primer ; sample)	350 steps/s
Wash/ flush	Repeat	1 times
	Flush syringe at waste pos.; wipe	Enabled; Disabled
	Peripump time	1000 ms
	Detergent time	0
	System airgap after flush	5 µl

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